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AN INVESTIGATION OF *NEISSERIA GONORRHOEAE* BY A RED CELL SENSITIZATION TECHNIQUE

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I. INTRODUCTION

Little progress has been made in our knowledge of the antigenic structure of the gonococcus. There is no unanimity as to whether antigenic variants exist within the group, or whether all strains are serologically identical.

The consensus of opinion is that no distinction may be drawn between gonococcal strains by simple complement-fixation or agglutination tests (Warren, 1921; Cook & Stafford, 1921; Thomsen & Vollmond, 1921; Torrey & Buckell, 1922; Tulloch, 1923; Segawa, 1932, Stokinger, Carpenter & Plack, 1944).

With an agglutinin-absorption technique Hermanies (1921) claimed to have demonstrated six 'distinct clear-cut immunologic types, having very little relation with one another' among 85 strains. Similarly, Thomsen & Vollmond (1921) were able to define four groups among 26 strains, and Miravent, Quiroga & Negroni (1926) four groups among 45 strains.

On the other hand, Torrey & Buckell (1922) were able to find no distinct serological types, but three ill-defined groups among 77 strains; Tulloch (1923) one large uniform group and a few ill-defined minor groups among 100 strains; and, finally, Segawa (1932) no serologically distinct types by agglutinin-absorption methods among the 64 strains investigated by him.

In this investigation an attempt has been made to apply a red cell sensitization technique, first mentioned in relation to the gonococcus by Thomas & Mennie (1950), to some of these problems.

II. METHODS

(a) *Strains*

All strains of *Neisseria gonorrhoeae* were freshly isolated from untreated cases of acute urethritis in males, and all the strains fulfilled the usual morphological, cultural, and biochemical characters of *N. gonorrhoeae*.

The different strains and the extracts prepared from them have been labelled alphabetically A, B, C, etc.; the corresponding antisera a, b, c, etc.

(b) *Media*

Heated blood (chocolate) agar containing 10–15% human blood laked by freezing and thawing was used for isolation and propagation of strains. Cultures were incubated at 37° C. for 18 hr. under 10% CO_2.

Biochemical reactions were investigated using Bacto phenol-red broth base with 1·25% agar, 30% ascitic fluid, and 1·0% of the appropriate sugar sterilized by Seitz filtration.

(c) *Preparation of sensitizing extract*

A growth from approximately 50 Petri dishes was suspended in 10 ml. saline and 0·5 ml. N/1 NaOH added. When solution was complete excess alkali was cautiously neutralized by the addition of N/1 HCl, 0·1 ml. at a time. Five volumes of absolute alcohol were added and the flocculent precipitate recovered by centrifugation.

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This precipitate was resuspended in 20 ml. saline (0·85 %), or in a phosphate buffer of pH 6·9, by shaking. The insoluble bulk was removed by centrifugation; the clear supernatant contained a soluble antigen which when adsorbed on to red cells sensitized them to the action of a gonococcal antiserum. For convenience the supernatant is referred to as the sensitizing extract. Occasional extracts proved slightly haemolytic for sheep cells and were unsuitable for use.

(*d*) *Preparation of antisera*

Rabbits were immunized intravenously as follows, using a suspension containing 10^9 organisms per ml.:

1st day 0·3 ml. of a heat-killed suspension
4th day 1·0 ml. of a live suspension
25th day 1·0 ml. of a live suspension

Subsequently the third injection was omitted and the animals were bled out a week after the second injection. Sera prepared by either method gave satisfactory titres in an agglutination test as well as in the gonococcal haemolysis test. A similar technique employed in preparing meningococcal antisera, while giving high titres when tested against red cells sensitized with the homologous extract, gave relatively low titres in an agglutination test.

It must be emphasized that the first injection was given as soon as the organism was obtained in pure culture, i.e. within 2–3 days of primary isolation.

All antisera were inactivated at 56° C. for 30 min. and absorbed with packed sheep cells before use in the gonococcal haemolysis test and in mirror absorption tests.

(*e*) *Sensitization of red cells*

The sensitizing extract was diluted with saline. The requisite dilution depended on the potency of the particular extract, but in general a 1 in 25 dilution was satisfactory. Packed sheep cells were added to make a 2·0 % suspension and this was incubated at 37° C. for 1 hr. The cells were then washed 3 times with saline, and finally resuspended in saline to make a 1·0 % suspension. This suspension of sensitized cells served as the 'antigen' in the gonococcal haemolysis test. In some of the earlier experiments a 0·5 % sensitized cell suspension was used.

Washing of the sensitized cells was necessary because the sensitizing extract contained soluble gonococcal antigens which though not adsorbable on to red cells could yet react with a gonococcal antiserum.

(*f*) *The gonococcal haemolysis test*

Serial doubling dilutions of the serum were made using 0·1 or 0·2 ml. volumes. To each tube was added an equal volume of the sensitized cell suspension, and an equal volume containing an excess of complement (generally a 1 in 15 dilution of guinea-pig serum). The rack was shaken and incubated for 1 hr. in a 37° C. water-bath.

The titre was taken as the reciprocal of the highest initial serum dilution showing

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clear, sparkling haemolysis. Negatives showed the usual small button or crescent of deposited cells.

The controls are set out in Table 1. None of these showed haemolysis. The normal cell suspension was a 1·0 % suspension of sheep cells of the batch used for sensitization in the test.

Table 1. *Controls in the gonococcal haemolysis test*

(Quantities in ml.)

	Serum	Saline	Sensitized cells	Normal cells	Complement excess
Serum control	0·1*	.	.	0·1	0·1
Sensitized cell control	.	0·1	0·1	.	0·1
Normal cell control	.	0·2	.	0·1	.
Complement control	.	0·1	.	0·1	0·1

* 0·1 ml. of the initial serum dilution was used

(*g*) *The absorption test*

The absorption was carried out in the usual way with packed sensitized cells replacing a bacterial suspension. Sensitized cells can be tested very simply, by addition of complement, to see whether they have in fact absorbed antibody from a serum. If cells used for reabsorption fail to haemolyse, the earlier absorption may be taken as complete.

The serum to be absorbed was diluted to give a titre of 64 to 256; a dilution of 1 in 250 was generally adequate.

The absorbing cells were sensitized in the usual way, 0·2 ml. of a sensitizing extract prepared from the absorbing strain being used to sensitize 0·2 ml. of packed sheep cells. After the final wash the supernatant saline was discarded, the deposited absorbing cells were resuspended in 2·5 ml. of the diluted serum, and the absorption allowed to proceed at 37° C. for 30 min. The suspension was then centrifuged and both the supernatant serum and the deposited cells were retained.

The serum was tested in the gonococcal haemolysis test using cells sensitized with a 1 in 25 dilution of either the homologous sensitizing extract, or, failing that, with a sensitizing extract prepared from a gonococcus of the same type (see below), in order to follow the decline in titre. This required 0·2 ml. of serum, the remainder being added to a second lot of absorbing cells and the process repeated.

Table 2. *Test for completeness of absorption of a serum by testing cells used for re-absorption*

(Quantities in ml.)

	Tube 1	Tube 2
Saline	0·3	0·7
Absorbing cells	0·1	0·1
Complement (excess)	0·4	.

After absorption the absorbing cells were resuspended in 10 ml. saline and two tubes set up as shown in Table 2. These were incubated at 37° C. for 1 hr. The

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absorption was repeated until the resuspended cells showed no haemolysis in such a test.

The absorption could readily be completed in a single stage by using a large volume of cells sensitized with a relative excess of sensitizing extract. However, in order to conserve the sensitizing extract it was preferred to repeat an absorption on the same serum with cells sensitized with relatively small volumes of extract.

(h) *The agglutination test*

This was carried out with a gonococcal suspension containing 10^9 organisms per ml. in Dreyer tubes. Tests were incubated at 56° C. for 18 hr.

(i) *The complement-fixation test*

Antigens were prepared by Price's (1933) method. In some of the experiments the sensitizing extract was employed as a complement-fixation antigen. Standard Kolmer methods were employed.

III. FACTORS CONCERNED IN THE ADSORPTION OF A SOLUBLE GONOCOCCAL ANTIGEN ON TO RED CELLS

As a preliminary investigation the various factors involved in the adsorption of an antigen on to red cells were investigated; these were, the concentration of the sensitizing extract, the time allowed for its adsorption, the temperature, the capacity of the red cells to take up antigen, and finally, as shown by Neter & Zalewski (1953), the presence of electrolytes. The sensitizing extract in these experiments was prepared from 15 pooled gonococcal strains.

(a) *Concentration of sensitizing extract*

In this experiment the amount of antigen adsorbed on to the red cells was varied by varying the dilution of sensitizing extract from 1 in a 100 to 1 in 25. The red cells can adsorb all the antigen to which they are exposed (see below) so that an increase in the concentration of the extract automatically increases the amount of antigen adsorbed.

Table 3. *The effect of variation in the dilution of the extract used in sensitizing the red cells on the titre of a serum in the gonococcal haemolysis test*

Dilution of sensitizing extract	Titre of a serum in the gonococcal haemolysis test					
	512	1024	2048	4096	8192	16384
1 in 100	+	+	±	−	−	−
1 in 90	+	+	+	±	−	−
1 in 70	+	+	+	+	−	−
1 in 50	+	+	+	+	±	−
1 in 25	+	+	+	+	±	−

+ = haemolysis; ± = partial haemolysis; − = no haemolysis.

Table 3 shows that as the strength of the sensitizing extract was increased from 1 in 100 to 1 in 70, the apparent titre of a serum in the gonococcal haemolysis test

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rose from 1024 to 4096. Further increase in concentration of sensitizing extract did not, however, produce a proportional rise in apparent titre: instead, a maximum was reached, which we may take as the true titre of the serum.

(b) *Variation in time of interaction*

Two dilutions of the sensitizing extract were made, 1 in 50 and 1 in 100. Cells were added to make the usual 2·0 % suspension, and each suspension was then divided into five portions, and placed in the 37° C. water bath. One tube from each batch was removed at ½, 1, 2, 3, and 5 hr. and the cells, washed and re-suspended in the usual way, were tested in the gonococcal haemolysis test.

Table 4 shows that maximum sensitization took place within half an hour and that prolonging the period of incubation to as long as 5 hr. did not produce a further rise in titre.

Table 4. *The effect of variation in the period of sensitization of cells on the titre of a serum in the gonococcal haemolysis test*

Time in water bath (hr.)	1 in 100 sensitized cells. Titres in the gonococcal haemolysis test			1 in 50 sensitized cells. Titres in the gonococcal haemolysis test		
	1024	2048	4096	4096	8192	16384
½	+	±	−	+	±	−
1	+	±	−	+	±	−
2	+	±	−	+	±	−
3	+	±	−	+	±	−
5	+	±	−	+	±	−

+ = haemolysis; ± = partial haemolysis; − = no haemolysis.

(c) *Effect of temperature*

A 2·0 % suspension of red cells was made in sensitizing extract diluted 1 in 50, one portion being left at 4° C. for 1 hr., and the other at 37° C. The cells were washed, resuspended in saline, and tested in the gonococcal haemolysis test (Table 5). Very little adsorption took place at the lower temperature.

Table 5. *The effect of temperature on the sensitization of red cells*

Temp. of sensitization	Titre of a serum in the gonococcal haemolysis test
4° C.	32
37° C.	4096

(d) *Capacity of red cells to adsorb antigen*

In this experiment a single batch of red cells was exposed to six successive portions of sensitizing extract. The effect of this repeated sensitization was investigated by testing a sample of the cells after each sensitization in the gonococcal haemolysis test. The supernatant fluid was tested for residual antigen by adding fresh cells to it and allowing sensitization to proceed in the usual way. The cells were then tested against the gonococcal antiserum.

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The detailed experiment was as follows:

Step 1. 0·1 ml. packed sheep cells were added to 5·0 ml. sensitizing extract diluted 1 in 50, and incubated at 37° C. for 30 min. The suspension was centrifuged and both cells and supernatant were retained.

(i) *Cells*. These were resuspended in 20 ml. saline, 2·0 ml. were removed, washed, and tested in the gonococcal haemolysis test.

(ii) *Supernatant*. To the 5·0 ml. supernatant 0·1 ml. packed sheep cells were added, the suspension was incubated, and the cells tested in the gonococcal haemolysis test after being washed and resuspended in 20 ml. saline.

Step 2. The 18 ml. cell suspension remaining from step 1 (i) was centrifuged, the supernatant saline was discarded, and 4·5 ml. fresh diluted sensitizing extract added to these already once sensitized cells. The suspension was incubated at 37° C. for 30 min., and centrifuged.

(i) *Cells*. The cells were suspended in 18 ml. saline and 2·0 ml. were removed for testing in the gonococcal haemolysis test.

(ii) *Supernatant*. To the 4·5 ml. supernatant was added 0·09 ml. packed sheep cells. After incubation these were washed, suspended in 18 ml. saline, and tested as described.

The same process was repeated in steps 3–6, the cell suspension being maintained during sensitization at 2·0 % and during testing at 0·5 %. The same antiserum was used throughout.

Table 6. *The effect of six sensitizations of a single batch of red cells on the gonococcal haemolysis test, and the estimation of residual antigen in the supernatant following each of these sensitizations.*

	Titre of a serum in the gonococcal haemolysis test							
	With cells sensitized with the supernatants			Following successive resensitization of a batch of red cells				
Sensitizations	Neat	2	4	1024	2048	4096	8192	16384
1	–	–	–	+	+	–	–	–
2	–	–	–	+	+	+	±	–
3	–	–	–	+	+	+	±	–
4	–	–	–	+	+	+	±	–
5	–	–	–	+	+	+	±	–
6	–	–	–	+	+	+	±	–

+ = haemolysis; ± = partial haemolysis; – = no haemolysis.

The results are set out in Table 6. The supernatant removed after sensitizing a batch of red cells contained no residual antigen capable of being adsorbed on to red cells and detectable in the gonococcal haemolysis test, even if the red cells used had been subjected to six previous sensitizations. The red cells had adsorbed enough antigen to react to titre with the serum after the second sensitization, but they were still able to take up all the additional antigen to which they were exposed in subsequent sensitizations, without, however, changing the titre of a serum titrated against them.

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(e) *Effect of electrolytes*

Neter & Zalewski (1953) showed that electrolytes were essential for adsorption of *Escherichia coli* antigens on to red cells. In repeating these experiments with a 5·0% dextrose solution replacing saline at all stages of the red cell sensitization procedure, it was found that the titre in the gonococcal haemolysis test was considerably lowered when sensitization was carried out in the absence of electrolytes, so that these may be regarded as essential for adequate sensitization.

To sum up, adsorption of a gonococcal antigen on to red cells took place rapidly at 37° C. and in the presence of electrolytes; all the available antigen in the solution was taken up. The only variable was the amount of antigen which was offered for adsorption.

IV. THE SMOOTH-ROUGH CHANGE IN THE GONOCOCCUS

(a) *Effect of the S-R change on the gonococcal haemolysis test*

These investigations were commenced in 1951 with fifteen gonococcal strains. The growth was pooled and a potent sensitizing extract prepared from them. The strains were then maintained on chocolate agar slopes in screw-capped bottles at 37° C. and subcultured at 7- to 10-day intervals.

After about 4 months on stock culture the organisms were again harvested and extracted. The antigen obtained after this period, however, failed to sensitize cells in the gonococcal haemolysis test. Although at first this failure was attributed to some inapparent change in technique, it soon became obvious that a change in the organisms was responsible. When the extraction was repeated with fresh gonococcal strains a potent antigen was readily obtained.

These observations have since been repeatedly confirmed with individual gonococcal strains.

Thus there is an antigen present in recently isolated gonococci which can be adsorbed on to red cells, and which sensitizes these cells to the action of a gonococcal antiserum. This antigen is lost on subculture and, under the conditions described, can generally no longer be detected after 4–6 months of cultivation. Its presence can still be demonstrated at this stage by immunizing animals and testing their sera with cells sensitized with a potent extract.

(b) *Effect of the S-R change on the agglutination test*

When it loses its surface antigen the gonococcus becomes relatively inagglutinable with sera prepared against smooth phase organisms, but with antisera prepared against rough strains high titres usually occur with old strains, and lower titres with smooth strains. These changes have been followed with several gonococcal strains; an example is shown in Table 7. Essentially similar observations have been recorded by Atkin (1925).

(c) *The morphological S-R change*

Tulloch (1923) writes: 'The most constant feature of the growth (of the gonococcus) is its peculiar mucus-like quality which is readily appreciated when a colony is picked off for isolation, for the growth tends to hang to the edge of the loop and

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Table 7. *The influence of the S-R change in the gonococcus on agglutinin titres*

Antiserum	Agglutinable suspensions G_0	G_3	G_6
b_0	1024	32	4
c_0	1024	32	32
d_0	256	128	64
e_0	512	16	16
f_0	4096	512	32
j_0	128	256	16
c_6	32	128	128
g_6	8	256	512
h_6	128	512	512
j_6	64	64	64

The figures 0, 3 and 6 in the designations of antisera and suspensions refer to the age of strain used in the preparation of each.

0 = recently isolated gonococcal strain used; 3 = gonococcal strain 3–4 months old; 6 = gonococcal strain 6 months old.

to the agar at the same time leaving quite an appreciable string of growth between loop and medium.'

This mucoid quality is characteristic of the smooth gonococcus and meningococcus, and appears to be due to some change on the surface of the organism on removal from the candle jar. This mucoid character of the colony becomes progressively more marked as the plate is left at room temperature, and with some strains the colony becomes so viscid that it cannot be picked off the agar. The rapidity of the change varies both with the culture medium, being more marked with chocolate agar than with blood agar, and with the strain, some becoming extremely difficult to manipulate after 10–15 min. on the bench; others show relatively little change after 24 hr.

With the change to the rough state the mucoid quality is gradually lost, the strain becoming drier and more easily manipulated. The same observation has been made by Oliver (1936).

V. CHEMICAL NATURE OF THE SENSITIZING EXTRACT

All the extracts prepared in the manner described gave a positive Molisch reaction, and negative biuret and xanthoproteic tests.

The heat stability of the sensitizing extract was investigated by heating a diluted extract in a 95° C. water-bath for 3 hr. Portions were removed periodically, cooled, and used to sensitize cells which were tested in the gonococcal haemolysis test. The results, set out in Table 8, show the antigen to be heat stable.

The antigen therefore appears to be heat stable and probably polysaccharide in nature.

VI. THE DEMONSTRATION OF TWO GONOCOCCAL TYPES

(*a*) *By a mirror cross-absorption technique*

Initially 18 strains of gonococci and the antisera prepared against them were compared by mirror cross-absorptions. If two strains were able to exhaust each others' antisera they were accepted as antigenically identical.

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Table 8. *The effect of heat on the activity of the sensitizing extract in the gonococcal haemolysis test*

Sensitizing extract heated at 95° C. for (hr.)	Titre of a serum in the gonococcal haemolysis test
Nil	4096
$\frac{1}{4}$	4096
$\frac{1}{2}$	4096
1	4096
$1\frac{1}{2}$	4096
2	4096
$2\frac{1}{2}$	4096
3	4096

The volume of sensitizing extract available limited the number of cross-absorptions that could be made; however, sufficient were made to indicate clearly the interrelationships between these strains. These results are set out in Table 9, and it will be seen that the 18 strains fall into two groups:

Type I. Fifteen strains which were able to exhaust all 18 antisera.

Type II. Three strains, E, I and P, which were able to exhaust each others' sera but not the sera corresponding to the 15 strains listed as type I.

Table 9. *The results of absorption tests with red cells sensitized with a gonococcal antigen using eighteen gonococcal strains and their antisera*

Gonococcal antigens	Gonococcal antisera																	
	b	c	d	e	f	h	i	j	k	m	n	p	r	s	t	u	v	w
B	–	–	–	.	.	–	–	.	.	.	.	.	.	.	.	.	.	.
C	.	–	.	.	.	–	.	–	.	.	.	.	.	.	.	.	.	.
D	–	–	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
E	+	+	.	–	.	+	–	.	.	.	+	–	.	+	.	.	.	.
F	–	–	–	.	.	–	–	.	.	.	.	.	.	.	.	.	.	.
H	.	–	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
I	+	+	.	–	.	+	.	.	.	.	+	–	.	+	.	.	.	.
J	.	–	–	.	.	.	.	.	.	–	–	.	–	–	–	–	–	–
K	.	–	.	.	.	.	.	.	.	.	.	.	–	–	–	–	–	–
M	.	–	.	–	–	–	.	–	–	.	.	–	–	–	–	–	–	–
N	.	–	.	–	.	.	.	.	.	.	.	.	–	–	–	–	–	–
P	.	+	.	–	.	+	–	.	.	.	+	–	.	+	.	.	.	.
R	.	.	.	.	.	.	–	–	.	.	.	.	.	.	.	.	.	.
S	.	.	.	.	.	.	–	–	.	.	.	.	.	.	.	.	.	.
T	.	.	.	.	.	.	–	.	–	.	.	.	.	.	.	.	.	.
U	.	.	.	.	.	.	–	.	–	.	.	.	.	.	.	.	.	.
V	.	.	.	.	.	.	–	.	–	.	.	.	.	.	.	.	.	.
W	.	.	.	.	.	.	–	.	–	.	.	.	.	.	.	.	.	.

– = antigen will absorb out the corresponding antiserum. + = antigen will not absorb out the corresponding antiserum.

The results obtained with some of these absorptions are set out in more detail in Tables 10 and 11. A type I serum (Table 10) was completely absorbed by type I sensitized cells but not by type II cells. A type II serum was completely absorbed by both gonococcal types (Table 11).

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Table 10. *Absorption of a type I gonococcal antiserum* (*n*) *by* (i) *a type I antigen* (*J*), (ii) *a type II antigen* (*E*)

Antiserum ...	Type I (n)			
Absorbing cells ...	Type I (J)		Type II (E)	
Stage	Serum titre	Absorbing cells	Serum titre	Absorbing cells
Initial	128	.	128	.
Absorption 1	8	+	64	+
Absorption 2	0	+	32	+
Absorption 3	0	−	32	±
Absorption 4	.	.	32	−

+ = haemolysis; ± = partial haemolysis; − = no haemolysis.

Table 11. *The absorption of a type II gonococcal antiserum* (*e*) *by* (i) *a type I antigen* (*N*), (ii) *a type II antigen* (*P*).

Antiserum ...	Type II (e)			
Absorbing cells ...	Type I (N)		Type II (P)	
Stage	Serum titre	Absorbing cells	Serum titre	Absorbing cells
Initial	256	.	256	.
Absorption 1	4	+	8	+
Absorption 2	0	+	0	+
Absorption 3	0	+	0	+
Absorption 4	0	−	0	−

+ = haemolysis; ± = partial haemolysis; − = no haemolysis.

(*b*) *By absorbed antisera*

The two gonococcal types can be readily distinguished in the gonococcal haemolysis test by using a type I serum which has been absorbed with a type II antigen.

An undiluted type I gonococcal antiserum was absorbed with a packed suspension of type II gonococci, and the serum, after absorption, tested against type I and type II sensitized cells. The results set out in Table 12 show that the absorbed serum did not react with type II sensitized cells, but did react to lower titre with type I cells.

Table 12. *The effect of absorption of a type I gonococcal antiserum* (*n*) *with a suspension of* Neisseria gonorrhoeae (*type II*) *on the titre in the gonococcal haemolysis test using type I and type II sensitized test cells*

	Titres in the gonococcal haemolysis test	
Type I antiserum (n)	Type I sensitized cells	Type II sensitized cells
Unabsorbed	8192	4096
Absorbed	512	0

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A type I serum which was absorbed with type II sensitized cells behaved in a similar manner. This is shown in Table 13. The sera were diluted 1 in 250 and, after absorption with type II sensitized cells, tested in the gonococcal haemolysis test against various type I and type II cells. The sera failed to react with type II cells, but did react with type I cells.

Table 13. *The differentiation of type I and type II gonococcal types by the gonococcal haemolysis test using a type I antiserum which had been absorbed out with type II sensitized cells.*

		Titres with					
	Absorbed with Type II cells	Type I sensitized cells. Antigen			Type II sensitized cells. Antigen		
Type I serum*	(E or I)	K	L	M	E	I	P
b	I	16	16	16	0	0	0
b	E	8	16	16	0	0	0
h	I	16	16	16	0	0	0
h	E	64	64	64	0	0	0
n	I	8	16	16	0	0	0
n	E	16	16	16	0	0	0

* Diluted 1 in 250.

These absorption tests show that the two gonococccal types share a common antigen. The type I gonococcus, however, must possess an additional antigen because a type I gonococcus can absorb out both a type I and a type II antiserum, whereas the type II gonococcus cannot complete the absorption of a type I antiserum. If the type I antigen is given the symbol AB the type II antigen will be represented by A.

The difference between the two types could not be shown by agglutination or complement-fixation tests, nor by the gonococcal haemolysis test using unabsorbed sera. The significance of titres in the gonococcal haemolysis test was complicated by the fact that it was possible to sensitize cells to react to titre with these sera (fully sensitized cells), or to react short of titre (partially sensitized cells). In the latter case the titres merely reflected the sensitizing potency of the various extracts and in no way indicated possible antigenic differences between them. The reading of results with partially sensitized cells was complicated by a type of prozone phenomenon that was encountered with certain sera. An example is given in Table 14. This was not encountered with fully sensitized cells.

(c) *Distribution of the two types*

Following the investigation of the 18 strains of gonococci by a mirror absorption technique and their division into two types, a larger series of gonococcal strains was investigated.

The technique was simplified in so far as extraction of the sensitizing extract was carried out on the growth from eight to ten Petri dishes (instead of fifty), using

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appropriately smaller volumes of reagents, and dissolving the final product in 5·0 ml. buffer. Cells sensitized with the extract were tested in absorption tests against antisera prepared with type I and type II gonococci.

In this manner altogether 67 strains were investigated. Of these 59 proved to be type I strains, and 8 type II strains.

Table 14. *A prozone phenomenon in the gonococcal haemolysis test*

Degree of sensitization	Tube*											
	1	2	3	4	5	6	7	8	9	10	11	12
1 in 50	+	+	+	+	+	+	+	+	+	+	+	±
1 in 100	+	+	+	+	+	+	+	+	+	+	+	±
1 in 150	+	+	+	+	+	+	+	+	+	+	+	±
1 in 200	+	+	+	+	+	+	+	+	+	+	+	±
1 in 250	+	+	+	+	+	+	+	+	+	+	+	±
1 in 300	−	+	+	+	+	+	+	+	+	+	+	±
1 in 350	−	−	±	+	+	+	+	+	+	+	+	±
1 in 400	−	−	−	±	+	+	+	+	+	+	±	−
1 in 450	−	−	−	±	+	+	+	+	+	+	±	−
1 in 500	−	−	−	−	±	±	+	+	+	±	−	−
1 in 550	−	−	−	−	−	±	+	+	+	±	−	−
1 in 600	−	−	−	−	−	±	±	+	+	±	−	−

* Serial doubling dilutions of serum (f) starting at 1 in 32.
+ = haemolysis; ± = partial haemolysis; − = no haemolysis.

VII. THE RELATIONSHIP TO THE MENINGOCOCCUS

Eight meningococcal strains were isolated from sporadic cases of meningitis. The growth from each of these strains was extracted in the same way as described for the gonococcus and antisera were prepared against them. Mirror absorptions with meningococcal sensitized cells showed that all these eight strains shared the identical antigen active in a 'meningococcal haemolysis test'.

Mirror absorptions with the meningococcal and gonococcal sensitizing extracts indicated that the antigen present in the meningococcus was identical with that present in the type I gonococcus.

VIII. THE RELATIONSHIP TO THE NON-PATHOGENIC NEISSERIAE

These strains were not as fully investigated as the other groups. The results, however, proved interesting, and it was decided to include them in this paper.

Twenty-eight strains were investigated. Four, *N. catarrhalis*, *N. sicca*, *N. haemolysans* and *N. flavus*, were obtained from the National Collection of Type Cultures, Colindale, London. The remainder were local strains isolated from the nasopharynx. Their biochemical behaviour was variable and they could not readily be assigned to any of the usual biochemical types.

The growth from each of these strains was extracted as described in the case of the gonococcus, and cells sensitized with the extracts were tested against a type I gonococcal antiserum.

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Fourteen of these extracts proved to be haemolytic for normal sheep cells and could not be tested further. Cells sensitized with the sensitizing extract of 13 strains did not react with the gonococcal antiserum.

Cells sensitized with the extract from one strain cross reacted strongly with the gonococcal antiserum. Unfortunately the strain was lost and further investigations could not be carried out.

IX. RELATIONSHIP BETWEEN THE ANTIGENS CONCERNED IN THE GONOCOCCAL AGGLUTINATION, COMPLEMENT-FIXATION, PRECIPITIN AND HAEMOLYSIS TESTS

(a) *The effect of absorbing sera with gonococcal sensitized cells*

In this experiment sera representing the two gonococcal types were absorbed with red cells sensitized with the homologous sensitizing extracts. Thus a type I serum (j) diluted 1 in 25 was absorbed with J sensitized cells, and a type II serum (i) diluted 1 in 20 was absorbed with I sensitized cells. The object was to investigate the effect of removal of antibodies against the sensitizing antigen on the complement-fixing and agglutinating activity of that serum.

The dilutions were selected to permit a reasonably easy absorption, and still give a positive complement-fixation titre of at least two to three tubes. Even so, 12 to 19 absorptions using 0·5 ml. sensitizing extract for each absorption, were required before the sera gave negative gonococcal haemolysis tests, and before the absorbing cells no longer haemolysed in the presence of complement. Activity of a serum in the gonococcal haemolysis test can be removed far more expeditiously by absorption with a suspension of gonococci. This procedure however not only removed all antibodies from the serum, but generally rendered the serum anti-complementary, a finding in conformity with that recorded by Reyn (1949).

The original unabsorbed serum and the absorbed serum were then tested in an agglutination and complement-fixation test. To facilitate comparison all the titres have been expressed in terms of an undiluted serum. The results, set out in Table 15, show that when all antibodies reacting in the gonococcal haemolysis test were removed, the absorbed serum showed little or no change in titre in an agglutination or complement-fixation test.

Table 15. *The results of the gonococcal haemolysis, agglutination, and complement-fixation tests with a type I serum (j) and a type II serum (i) before and after absorption of these sera with homologous sensitized red cells.*

		Titres with antigen J using		Titres with antigen I using	
	Sera	Unabsorbed serum	Absorbed serum	Unabsorbed serum	Absorbed serum
Gonococcal haemolysis test	j	16384	0	8192	0
	i	16384	0	4096	0
Agglutination test	j	400	400	200	200
	i	160	320	1280	1280
Complement-fixation test	j	100	200	25	25
	i	40	40	40	20

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(b) Sensitizing extract and the complement-fixation test

It has been demonstrated that exposure of the sensitizing extract to sheep cells will completely remove the adsorbable antigen active in the gonococcal haemolysis test. The extract after absorption will no longer sensitize cells in the gonococcal haemolysis test. This experiment was carried out to determine the complement-fixing properties of the sensitizing extract before and after such an absorption.

A 1 in 10 dilution of sensitizing extract was made. A portion of this was retained and is referred to as the 'unabsorbed extract'. A second portion was absorbed with a one-fifth volume of packed sheep cells for 1 hr. at 37° C., and is referred to as the 'absorbed extract'.

The unabsorbed and the absorbed extracts were then tested for their capacity to sensitize cells in the gonococcal haemolysis test; for their complement-fixing activity by using them as antigen in a titration; and for anti-complementary activity.

Table 16. *The effect of removal of the antigen active in the gonococcal haemolysis test from a sensitizing extract on the complement-fixing activity of that extract, and on its anti-complementary activity.*

	Titre of a serum in the gonococcal haemolysis test		Complement-fixing activity		Anti-complementary activity	
Sensitizing extract	Unabsorbed extract	Absorbed extract	Unabsorbed extract	Absorbed extract	Unabsorbed extract	Absorbed extract
I	4096	0	0	0	0	0
J	16384	0	1280	1280	0	0
K	32768	0	640	320	0	0
M	16384	0	1280	1280	0	0
N	16384	0	1280	1280	0	0
R	4096	0	0	0	0	0
S	16384	0	0	0	0	0
T	8192	0	40	40	0	0
U	16384	0	0	0	0	0

* The complement-fixing activity has been expressed as the highest initial dilution of the sensitizing extract which fixed complement.

The results are set out in Table 16 and they bring out a number of points.

(i) Different sensitizing extracts differ in their complement-fixing activity, which varies from nil to 1 in 1280.

(ii) The complement-fixing activity of the extract is uninfluenced by removing the adsorbable antigen on to red cells (sensitizing extracts J, K, M, N and T).

(iii) The antigen active in the gonococcal haemolysis test appears to have no complement-fixing activity (the unabsorbed I, R, S and U extracts).

The anti-complementary titration serves as a control.

The sensitizing extract therefore contains at least two demonstrable antigens. The one, adsorbable on to red cells, is detected in the gonococcal haemolysis test; the other is active in a complement-fixation test.

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(c) Blocking effect of absorbed sensitizing extract on the gonococcal haemolysis test

The presence of an antigen in a sensitizing extract from which the adsorbable antigen has been absorbed completely with sheep cells, can be demonstrated in another manner.

Serial doubling dilutions of the absorbed sensitizing extract were made (Table 17). To each tube was added 0·2 ml. of a 1 in 50 dilution of a sheep-cell-absorbed and inactivated gonococcal antiserum. Serum and sensitizing extract controls were included. The rack was then incubated for 30 min. at 37° C. In this way the antigen present in the absorbed sensitizing extract was allowed to react directly with the serum. In order to detect this reaction gonococcal sensitized cells and complement were added as in the gonococcal haemolysis test, and the rack read for haemolysis after 1 hr. in the 37° C. water-bath. The usual controls for the gonococcal haemolysis test were included.

Table 17. *The blocking effect on the gonococcal haemolysis test of the antigen present in the supernatant after the sensitization of red cells*

(Quantities in ml.)

Tube	'Absorbed' sensitizing extract, 0·2 ml.	Antiserum 1 in 50		Sensitized cells	Complement excess		Result
1	Neat	0·2	37° C. for 30 min.	0·2	0·2	37° C. for 1 hr.	–
2	1 in 2	0·2		0·2	0·2		–
3	1 in 4	0·2		0·2	0·2		–
4	1 in 8	0·2		0·2	0·2		±
5	1 in 16	0·2		0·2	0·2		+
6	1 in 32	0·2		0·2	0·2		+
7	1 in 64	0·2		0·2	0·2		+
8	1 in 128	0·2		0·2	0·2		+
9	1 in 256	0·2		0·2	0·2		+
10	1 in 512	0·2		0·2	0·2		+
11	Saline*	0·2		0·2	0·2		+
12	Neat	Saline*		0·2	0·2		–

* 0·2 ml. saline

+ = haemolysis; ± = partial haemolysis; – = no haemolysis.

The results in the last column of Table 17 show an inhibition of haemolysis in the lower dilutions of the absorbed sensitizing extract.

In Table 18 the complement-fixing and blocking titres of seventeen such absorbed sensitizing extracts are compared. Those extracts which were unable to fix complement were also unable to block the action of a serum on gonococcal sensitized cells. There is further a rough correlation between the complement-fixing titres of these extracts and their blocking ability. Extracts with complement-fixing titres of 20 had only partial blocking activity in the first tube which contained a 1 in 10 dilution of extract, or no blocking activity at all. Those with greater complement-fixing titres produced a correspondingly more marked blocking effect. It seems probable, therefore, that the same antigen is concerned in these two tests.

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(d) *Precipitin tests*

No precipitation could be demonstrated between various sensitizing extracts and antisera, using both a layering and a constant antibody optimal proportions technique.

(e) *Antigenicity of the sensitizing extract*

The sensitizing extract proved a potent antigen producing antisera active in the gonococcal haemolysis, agglutination, and complement-fixation tests.

Immunization of rabbits with a sensitizing extract which had been absorbed with sheep cells and hence lacked the antigen which sensitizes cells in the gonococcal haemolysis test, still produced antisera active in the haemolysis as well as

Table 18. *Comparison of the complement-fixing and blocking activity of various 'absorbed' sensitizing extracts*

'Absorbed' sensitizing extract	Complement-fixing activity	Blocking activity on the gonococcal haemolysis test
1	40	10
2	0	0
3	20	± 10
4	0	0
5	0	0
6	0	0
7	0	0
8	0	0
9	0	0
10	80	20
11	80	10
12	20	± 10
13	320	80
14	40	20
15	20	0
16	20	± 10
17	160	40

± = Partial blocking in first tube.

complement-fixation tests. Thus antigenic groupings identical with that active in the gonococcal haemolysis test were still present in the absorbed sensitizing extract. This observation is in keeping with the blocking effect of such an extract on the gonococcal haemolysis test.

There appeared to be some correlation between the complement-fixing titre of an extract and its toxicity to rabbits. Thus sensitizing extracts J and N with titres of 1280 (Table 16) proved so toxic that attempts to produce antisera with them were abandoned. Extract 10 (Table 18) proved fatal to three of nine rabbits in a dose of 0·005 ml. Its complement-fixing titre was 80. On the other hand, extracts E and I which had no complement-fixing activity proved non-toxic and a dose of 0·5 ml. was well tolerated.

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X. DISCUSSION

The surface of the smooth-phase gonococcus contains a polysaccharide complex which is lost with the S-R change. Two antigens may be demonstrated on extraction of these smooth-phase organisms.

The one, active in the gonococcal haemolysis test, behaves like a simple haptene, failing to react in a precipitin test, and not fixing complement in the presence of an immune serum. The second antigen appears to be primarily responsible for complement fixation.

It is suggested that the antigen active in the gonococcal haemolysis test, functioning as a determinant haptene, is linked with the complement-fixing antigen as a complex antigen on the surface of the gonococcus, and that the haptene is partially split off in the preparation of the sensitizing extract. The presence of antigenic groupings in the complement-fixing antigen similar to that present in the sensitizing haptene is indicated by the effect of animal inoculation with an absorbed extract when antibodies in the haemolysis test are produced. The presence of such groupings is also shown by the blocking action of an absorbed extract on the activity of a serum on gonococcal sensitized cells.

That the sensitizing haptene, however, is not the only determinant in this antigenic complex seems probable from the result of absorption of sera with sensitized cells. Here removal of all antibodies active in the gonococcal haemolysis test did not materially interfere with the complement-fixing and agglutinating activity of that serum.

The application of the gonococcal haemolysis test to gonococcal infection in man is outside the scope of this paper and has not been discussed. One point is relevant, however, and that is that approximately 30% of patients with simple gonococcal urethritis give a positive gonococcal haemolysis test on first presenting for treatment, so that the sensitizing haptene does function as a not unimportant determinant in natural infection with the gonococcus. A positive gonococcal haemolysis test was also obtained in a case with meningococcal infection.

In the case of the gonococcus this haptene is of at least two types. Eight meningococcal strains possessed the same antigen as that present in the type I gonococcus. The presence of a similar antigen in a non-pathogenic Neisseria from the naso-pharynx was curious. Can this have been the only smooth strain among the twenty-eight examined?

The failure of the antigen active in the gonococcal haemolysis test to fix complement proved convenient in so far as it enabled a ready distinction between the sensitizing antigen and the complement-fixing antigen to be made. Goodner & Horsfall (1936) have recorded the failure of a haptene-antibody system to fix complement.

Finally, the S-R change in the gonococcus is of some importance in interpreting the variable results obtained by previous workers in attempting to classify gonococci into serological types. With a few notable exceptions the majority of workers employed strains which had been under cultivation for considerable periods, and it seems probable that many of these strains were no longer in the smooth phase.

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SUMMARY

Two antigens, probably polysaccharide in nature, were extracted from smooth gonococci.

One of these could be adsorbed on to red cells and sensitized these cells to the action of a gonococcal antiserum. The factors concerned in this adsorption were investigated. The antigen, which was shown by a mirror absorption technique to be of two distinct types, was lost with the S-R change.

The second antigen was detected by its ability to fix complement in the presence of an immune serum.

It is suggested that these two antigens form a complex which determines the nature of the surface of the smooth gonococcus.

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